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(54) **VACCINE FOR PROTECTION AGAINST *LAWSONIA INTRACELLULARIS*, *MYCOPLASMA HYOPNEUMONIAE* AND PORCINE CIRCO VIRUS**

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See application file for complete search history.

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(57) **ABSTRACT**

The present invention pertains to a vaccine comprising in combination non-live antigens of *Lawsonia intracellularis*, of *Mycoplasma hyopneumniae* and Porcine circo virus, and a pharmaceutically acceptable carrier. The invention also pertains to a kit comprising a first container having contained therein non-live antigens of *Lawsonia intracellularis*, one or more other containers having contained therein *Mycoplasma hyopneumoniae* and Porcine circo virus antigens and instructions for mixing the antigens of *Lawsonia intracellularis, Mycoplasma hyopneumoniae*, and Porcine circo virus to formulate one combination vaccine suitable for systemic vaccination.

6 Claims, No Drawings

VACCINE FOR PROTECTION AGAINST *LAWSONIA INTRACELLULARIS, MYCOPLASMA HYOPNEUMONIAE* AND PORCINE CIRCO VIRUS

REFERENCE TO RELATED APPLICATIONS

This application is a Divisional of U.S. application Ser. No. 12/988,088, filed Oct. 15, 2010, which is a national stage entry under 35 U.S.C. §371 of PCT/EP2009/054517, filed on Apr. 16, 2009, which claims priority to U.S. Provisional Application No. 61/046,188, filed on Apr. 18, 2008, and EP Application No. 08154765.5, filed on Apr. 18, 2008. The content of which is hereby incorporated by reference in its entirety.

The present invention pertains to a vaccine for protection against *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine circo virus. Protection in this sense means that the vaccine at least provides a decrease in a negative influence caused by *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine circo virus, such negative influence being e.g. tissue damage and/or clinical signs such as decreased weight gain, diarrhea, coughing, sneezing etc. The present invention also pertains to a kit comprising a first container having contained therein non-live antigens of *Lawsonia intracellularis*, one or more other containers having contained therein *Mycoplasma hyopneumoniae* and Porcine circo virus antigens and instructions for mixing the antigens of *Lawsonia intracellularis, Mycoplasma hyopneumoniae*, and Porcine circo virus to formulate one combination vaccine suitable for systemic vaccination.

Proliferative enteropathy (PE or PPE, also called enteritis or ileitis) in many animals, in particular pigs, presents a clinical sign and pathological syndrome with mucosal hyperplasia of immature crypt epithelial cells, primarily in the terminal ileum. Other sites of the intestines that can be affected include the jejunum, caecum and colon. Weanling and young adult pigs are principally affected with typical clinical manifestation of rapid weight loss and dehydration. Natural clinical disease in pigs occurs worldwide. The disease is consistently associated with the presence of intracellular curved bacteria, presently known as *Lawsonia intracellularis*.

Mycoplasmal pneumonia of swine caused by the bacterial pathogen *Mycoplasma hyopneumoniae* is a widespread chronic respiratory disease in pigs. Especially young piglets are vulnerable to this, non-fatal, disease. The enzootic pneumonia is a chronic disease that results in poor feed conversion and stunted growth. The disease is highly contagious and transmission is usually through direct contact with infected respiratory tract secretions, e.g. in the form of infected droplets after coughing/sneezing. The most problematic consequence of this disease is that it predisposes for all kinds of secondary infections of the respiratory system. It is estimated that e.g. in the USA, 99% of all pig farms are infected.

Porcine circo virus is thought to be linked to the post-weaning multisystemic wasting syndrome (PMWS) observed in young pigs. This disease was encountered for the first time in Canada in 1991.The clinical signs and pathology were published in 1997 and include progressive wasting, dyspnea, tachypnea, and occasionally icterus and jaundice. Porcine circo virus is a small (17 nm) icosahedral non-enveloped virus containing a circular single stranded DNA genome. PDNS (porcine dermatitis and nephropathy syndrome) is another major problem for pig farmers which appeared around the same time as PMWS and which is also related to Porcine circo virus. Characteristic of PDNS are red/brown circular skin lesions with haemorrhages, usually on the ears, flanks, legs and hams.

With respect to PE, oral vaccination against *Lawsonia intracellularis* has shown to be an economically efficient measure to control Ileitis and to allow a better exploitation of the genetic growth potential of the pig (Porcine Proliferative Enteropathy Technical manual 3.0, July 2006; available from Boehringer Ingelheim). Furthermore, oral rather than parenteral vaccination will reduce the transmission of blood-borne infections such as PRRS via multi-use needles and the reduction of injection site reactions and needles retained in carcasses. It will reduce animal and human stresses, time, labour costs and effort compared to individual vaccination (McOrist: "Ileitis—One Pathogen, Several Diseases" at the IPVS Ileitis Symposium in Hamburg, Jun. 28, 2004).

It is generally understood that the advantage of an attenuated live vaccine approach is that the efficacy of immunity is usually relatively good, as the host's immune system is exposed to all the antigenic properties of the organism in a more "natural" manner. Specifically for intracellular bacterial agents such as *Lawsonia intracellularis*, the live attenuated vaccine approach is believed to offer the best available protection for vaccinated animals, due to a full and appropriate T cell based immune response. This is in contrast with the variable to poor immunity associated with subunit or killed vaccine types for intracellular bacteria. This is also specifically true for obligate intracellular bacteria such as *Lawsonia intracellularis* or the *Chlamydia* sp, which cause pathogenic infections within mucosa. Studies indicate that whole live attenuated forms of the intracellular bacteria in question are best delivered to the target mucosa, that they are required as whole live bacterial forms to produce a fully protective immune response in the target mucosa but also that they are immunologically superior compared to use of partial bacterial components.

It has become a general understanding that a vaccine against *Lawsonia intracellularis* needs to be administered orally (see La. Technical Manual 3.0 as referred to here-above). This is based on the fact that the basis of the body's resistance to Ileitis is the local immunity in the intestine, which is the product of cell-mediated immunity and local defense via antibodies, especially IgA. According to current knowledge, serum antibodies (IgG) do not give any protection simply because they do not reach the gut lumen. It has been demonstrated in studies that oral vaccination produces cell-mediated immunity as well as local production of IgA in the intestine (Murtaugh, in Agrar-und Veterinär-Akademie, Nutztierpraxis Aktuell, Ausgabe 9, Jun. 2004; and Hyland et al. in Veterinary Immunology and Immunopathology 102 (2004) 329-338). In contrast, intramuscular administration did not lead to protection. Moreover, next to the general understanding that a successful vaccine against intracellular bacteria has to induce cell-mediated immunity as well as the production of local antibodies, the skilled practitioner knows that only a very low percentage of orally ingested antigens are actually absorbed by the enterocytes, and that the incorporation of *Lawsonia intracellularis* into the cell is an active process initiated by the bacterium. Accordingly an inactivated vaccine would provide the intestine with insufficient immunogenic antigen (Haesebrouck et al. in Veterinary Microbiology 100 (2004) 255-268). This is why it is believed that only attenuated live vaccines induce sufficient cell-mediated protection in the intestinal cells (see Technical Manual 3.0 as referred to here-above). At present there is only one vaccine on the market to protect against *Lawsonia intracel-*

lularis, viz. Enterisol® Ileitis marketed by Boehringer Ingelheim. This vaccine is a live vaccine for oral administration indeed.

Hitherto combination vaccines of *Lawsonia intracellularis* have been suggested in the prior art. However, not many of such combinations have actually been tested for efficacy. The reason for this is that it is generally understood that combination of antigens with antigens of *Lawsonia intracellularis* can only lead to successful protection if the *Lawsonia* antigens are provided as live (attenuated) cells. In this respect, we refer to WO 2005/011731, which also suggests all kinds of combination vaccines based on *Lawsonia intracellularis*. However, regarding the description and claim structure the patent application, the assignee (Boehringer Ingelheim) appears to be convinced that combination vaccines are only expected to have a reasonable chance of success when the *Lawsonia* antigens are present in the form of live cells. The same is true for WO2006/099561, also assigned to Boehringer Ingelheim. Indeed, based on the common general knowledge this is an obvious thought. Combination of live antigens however is not straightforward given the high chance of interference between the antigens and the difficulty of manufacturing such a live combination vaccine.

It is an object of the present invention to provide a vaccine to combat *Lawsonia intracellularis*, and at the same time combat one or more other swine pathogens. To this end a vaccine has been devised that comprises in combination non-live antigens of *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine circo virus, and a pharmaceutically acceptable carrier. Surprisingly, against the persistent general understanding how to combat *Lawsonia intracellularis* and that a combination vaccine should comprise live *Lawsonia intracelluaris* antigens, it was found that by using non-live *Lawsonia intracellularis* antigens, in combination with antigens from *Mycoplasma hyopneumoniae* and Porcine circo virus, a vaccine can be provided that protects against *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine circo virus.

In general, a vaccine can be manufactured by using art-known methods that basically comprise admixing the antigens with a carrier. Typically the antigen(s) are combined with a medium for carrying the antigens, often simply referred to as a carrier or "pharmaceutically acceptable carrier". Such a carrier can be any solvent, dispersion medium, coating, antibacterial and antifungal agent, isotonic and absorption delaying agent, and the like that are physiologically compatible with and acceptable for the target animal, e.g. by being made i.a. sterile. Some examples of such carrying media are water, saline, phosphate buffered saline, bacterium culture fluid, dextrose, glycerol, ethanol and the like, as well as combinations thereof. They may provide for a liquid, semi-solid and solid dosage form, depending on the intended mode of administration. As is commonly known, the presence of a carrying medium is not essential to the efficacy of a vaccine, but it may significantly simplify dosage and administration of the antigen. As such, the manufacturing of the vaccine can take place in an industrial environment but also, the antigens could be mixed with the other vaccine constituents in situ (i.e. at a veterinaries', a farm etc.), e.g. (immediately) preceding the actual administration to an animal. In the vaccine, the antigens should be present in an immunologically effective amount, i.e. in an amount capable of stimulating the immune system of the target animal sufficiently to at least reduce the negative effects of a post-vaccination challenge with wild-type micro-organisms. Optionally other substances such as adjuvants, stabilisers, viscosity modifiers or other components are added depending on the intended use or required properties of the vaccine.

In an embodiment, the vaccine is in a form suitable for systemic administration. To applicants surprise it has been found that one can induce a protection against *Lawsonia intracellularis* that is comparable with or even improved with respect to the protection provided by using the (single) live vaccine ENTERISOL® ILLEITIS (administered according to the corresponding instructions), when the combination vaccine according to the present invention is administered systemically, i.e. in a way that it reaches the circulatory system of the body (comprising the cardiovascular and lymphatic system), thus affecting the body as a whole rather than a specific locus such as the gastro-intestinal tract. Systemic administration can be performed e.g. by administering the antigens into muscle tissue (intramuscular), into the dermis (intradermal), underneath the skin (subcutaneous), underneath the mucosa (submucosal), in the veins (intravenous) etc. For systemic vaccination many forms are suitable, in particular liquid formulations (with dissolved, emulsified or suspended antigens) but also solid formulations such as implants or an intermediate form such as a solid carrier for the antigen suspended in a liquid. Systemic vaccination, in particular parenteral vaccination (i.e. not trough the alimentary canal), and suitable (physical) forms of vaccines for systemic vaccination have been known for more than 200 years. An advantage of this embodiment is that the same way of administration can be used that is the current standard for administering *Mycoplasma hyopneumoniae* or Porcine circo virus antigens, viz. parenteral, in particular via intramuscular or intradermal injection (in the latter case often needle-less).

In an embodiment the non-live *Lawsonia intracellularis* antigens are obtained from a carbohydrate containing composition, the carbohydrate being also found in live *Lawsonia intracellularis* cells in association with the outer cell membrane of these cells. Unexpectedly, by using a carbohydrate containing fraction of *Lawsonia intracellularis* cells (i.e. a composition containing the carbohydrates as present in live *Lawsonia qintracellularis* cells) in the combination vaccine, good protection against PE could be provided. It is noted that for formulating the vaccine a carbohydrate containing composition directly obtained from *Lawsonia intracellularis* cells could be used but also a composition derived therefrom, such as a dilution or concentrate of the original composition or an extract, one or more purified components etc. It is noted that subunits of *Lawsonia intracellularis* cells have been reported as antigens in a vaccine for protection against this bacterium. However, these are mainly recombinant proteins and hitherto none of them has proven to be able and provide good protection. It is also noted that a carbohydrate containing composition, wherein the carbohydrate is also found in live *Lawsonia intracellularis* cells in association with the outer cell membrane of these cells, is known from Kroll et al. (Clinical and Diagnostic Laboratory Immunology, June 2005, 693-699). However, this composition is used for diagnostics. It has not been tested as a protective antigen for reasons as stated hereabove.

In an embodiment, the carbohydrate containing composition is material resulting from the killing of *Lawsonia intracellularis* bacteria. It has been found that a very convenient way of providing the carbohydrate for use according to the present invention is to simply kill *Lawsonia intracellularis* cells and use the material resulting from that as a source for the carbohydrate. To extract the carbohydrate from living cells could in theory also be done (analogous to the creation of living ghost cells by removing the cell wall) but requires more sophisticated and thus more expensive techniques. The mate-

rial as a whole could be used, e.g. a suspension of whole cells or a lysate of *Lawsonia intracellularis* cells, or one could purify or even isolate the carbohydrate out of the material. This method can be performed by using relatively simple art-known techniques.

In a preferred embodiment the carbohydrate containing composition contains whole cells of killed *Lawsonia intracellularis* bacteria. This has proven to be the most convenient way to provide the carbohydrate as an antigen in the vaccine. Besides, the efficacy of the vaccine is even further increased, possibly since this way of offering the antigen to the immune system of the target animal better mimics the natural environment of the carbohydrate.

In an embodiment the vaccine comprises an oil in water adjuvant containing oil droplets of sub-micrometer size. In general, an adjuvant is a non-specific immunostimulating agent. In principal, each substance that is able to favor or amplify a particular process in the cascade of immunological events, ultimately leading to a better immunological response (i.e. the integrated bodily response to an antigen, in particular one mediated by lymphocytes and typically involving recognition of antigens by specific antibodies or previously sensitized lymphocytes), can be defined as an adjuvant. It has been shown that using an oil in water adjuvant containing oil droplets of sub-micrometer size provides a very good protection against *Lawsonia intracellularis*. Indeed, the application of oil in water adjuvants as such is common in connection with non-live antigens. However, it is generally known that the best immunostimulating properties are obtained when the oil droplets are large in diameter. In particular, oil droplets with a diameter beneath 1 micrometer are in particular used when it is believed that safety is an important issue. In that case, one could use small droplets since these are known to evoke less tissue damage, clinical signs etc. However, in the case of obtaining protection for a gut associated disorder via systemic vaccination (as is the case in the present invention), one would choose large droplets since one would expect that the immune response has to be boosted significantly. In contrast, we found that using small oil droplets in the composition provided very good results with respect to protection against *Lawsonia intracellularis*.

In an even preferred embodiment, the adjuvant comprises droplets of biodegradable oil and droplets of mineral oil, the droplets of biodegradable oil having an average size that differs from the average size of the droplets of mineral oil. It has been shown that the use of a mixture of biodegradable oil and mineral oil provides very good results with regard to efficacy and safety. In addition to this, stability of the composition is very high, which is an important economic advantage. The stability has proven to be very good, in particular when the average (volume weighed) size of either the biodegradable oil droplets or the mineral droplets is below 500 nm (preferably around 400 nm).

The present invention also pertains to a kit comprising a first container having contained therein non-live antigens of *Lawsonia intracellularis*, one or more other containers having contained therein *Mycoplasma hyopneumoniae* and Porcine circo virus antigens and instructions for mixing the antigens of *Lawsonia intracellularis, Mycoplasma hyopneumoniae*, and Porcine circo virus to formulate one combination vaccine suitable for systemic vaccination. In this embodiment, a separate container for the *Lawsonia intracellularis* antigens is provided in a kit containing also the other antigens (which are either combined in one container as known from the prior art or even present in separate containers that form part of the contents of the kit. An advantage of this embodiment is that the *Lawsonia* antigens can be prevented from having interactions with the other antigens until right before administration of the vaccine. Also, since the antigens are in a separate container less production losses will occur. In an embodiment the *Mycoplasma hyopneumoniae* and Porcine circo virus antigens are contained in one container, formulated in an oil in water adjuvant. In this embodiment, the *Lawsonia* antigens can be mixed with the other ones just before use.

The invention will be further explained based on the following examples.

Example 1 describes a method to obtain a substantially protein free carbohydrate containing composition and a vaccine that is made by using this composition.

Example 2 describes an experiment wherein a non-live *Lawsonia intracellularis* vaccine is compared with the vaccine currently on the market and an experimental vaccine comprising subunit proteins of *Lawsonia intracellularis*.

Example 3 describes an experiment wherein two different vaccines comprising non-live *Lawsonia intracellularis* antigens are compared with the live vaccine currently on the market.

Example 1

In this example a method is described to obtain a substantially protein free carbohydrate composition associated with the outer cell membrane of *Lawsonia intracellularis* cells and a vaccine that can be made using this composition. In general, a carbohydrate is an organic compound that contains carbon, hydrogen, and oxygen, usually in the ratio 1:2:1. Examples of carbohydrates are sugars (saccharides), starches, celluloses, and gums. Usually they serve as a major energy source in the diet of animals. *Lawsonia intracellularis* is a gram negative bacterium, which thus contains an outer membrane that is not constructed solely of phospholipid and protein, but also contains carbohydrates, in particular polysaccharide (usually polysaccharides such as lipopolysaccharide, lipo-oligosaccharide, or even non-lipo polysaccharides).

Carbohydrate Fraction for Vaccine Preparation

Twenty milliliters of buffered water (0.04 M PBS, phosphate buffered saline) containing *Lawsonia intracellularis* cells at a concentration of 3.7E8 (=3.7×10^8) cells/ml was taken. The cells were lysed by keeping them at 100° C. for 10 minutes. Proteinase K (10 mg/ml) in 0.04 M PBS was added to a final concentration of 1.7 mg/ml. This mixture was incubated at 60° C. for 60 minutes in order to degrade all proteins and keep the carbohydrates intact. Subsequently, the mixture was incubated at 100° C. for 10 minutes to inactivate the Proteinase K. The resulting material, which is a carbohydrate containing composition, in particular containing the carbohydrates as present in live *Lawsonia intracellularis* bacteria in association with their outer cell membrane (see paragraph below), was stored at 2-8° C. until further use. The composition was formulated in DILUVAC FORTE adjuvant which also serves as a carrier for the antigens. This adjuvant (see also EP 0 382 271) comprises 7.5 weight percent vitamine E acetate droplets with an average volume weighted size of approximately 400 nm, suspended in water and stabilized with 0.5 weight percent of TWEEN 80 (polyoxyethylene sorbitan mono-oleate). Each milliliter vaccine contained material that had been extracted from 1.2E8 *Lawsonia intracellularis* cells.

Immune Precipitation of *Lawsonia* Carbohydrate Antigens

Two batches of monoclonal antibodies (MoAb's) raised against whole cell *Lawsonia intracellularis* were precipitated with saturated Na_2SO_4 at room temperature according to standard procedures. The precipitate was pelleted by centrifu-

gation (10.000 g for 10 minutes). The pellet was washed with 20% Na_2SO_4 and resuspended in 0.04 M PBS. TYLOSYL activated DYNAL beads (DynaBeads, DK) were pre washed with 0.1 M $NaPO_4$ (pH 7.4), according the manual of the manufacturer. Of each batch of MoAb's 140 μg was taken and added to 2E8 pre washed beads and incubated overnight at 37° C. The beads were pelleted by centrifugation and non-bound MoAb's were removed by aspiration of the supernatant. Spectrophotometrical measurements showed that between 20 and 35% of the added MoAb's had bound to the beads. Two batches of 1 ml *Lawsonia intracellularis* cells (3.7E8/ml) in 0.04 M PBS were sonicated for 1 minute. The resulting cell lysates were added to the TYLOSYL activated beads—monoclonal complexes and incubated overnight at 4° C. The TYLOSYL activated beads—monoclonal complexes were washed three times with 0.1 M $NaPO_4$ (pH 7.4). The bound compounds were eluted by washing the beads in 0.5 ml 8M urea in 0.04 M PBS (E1); 0.5 ml 10 mM Glycine pH 2.5 (E2); and 0.5 ml 50 mM HCl (E3), in a sequential manner. After elution E2 and E3 were neutralized with either 100 μl and 200 μl 1 M Tris/HCl (pH8.0).

Samples were taken from each step and loaded onto SDS-PAGE gels. Gels were stained using Commassie Brilliant Blue (CBB) and Silver staining or blotted. The blots were developed using the same MoAb's as mentioned here-above. Inspection of the gels and blots showed that the MoAb's recognized bands with an apparent molecular weight of 21 and 24 kDa that were not seen on the CBB gels but were visible on de Silver stained gels. Also, it was established that the fraction of the cells that bound to the MoAb's was Proteinase K resistant. Thus, based on these results it can be concluded that this fraction contains carbohydrates (namely: all protein is lysed, and sonified DNA fractions will not show as a clear band in a Silver stain), and that the carbohydrates are in association with (i.e. forming part of or being bound to) the outer cell membrane of *Lawsonia intracellularis* (namely: the MoAb's raised against this fraction also recognized whole *Lawsonia intracellularis* cells). Given the fact that *Lawsonia intracellularis* is a gram-negative bacterium, the carbohydrate composition is believed to comprise polysaccharide(s).

Example 2

This experiment was conducted to test a convenient way to formulate the carbohydrate antigen in a vaccine, viz. via a killed whole cell (also known as bacterin). As controls the commercially available vaccine ENTERISOL® ILEITIS and an experimental subunit vaccine comprising protein subunits were used. Next to this unvaccinated animals were used as a control.

Experimental Design of Example 2

An inactivated whole cell vaccine was made as follows. Live *Lawsonia intracellularis* cells derived from the intestines of pigs with PPE were gathered. The cells were inactivated with 0.01% BPL (beta-propiolactone). The resulting material, which inherently is a non-live carbohydrate containing composition in the sense of the present invention (in particular since it contains the carbohydrates as present in live *Lawsonia intracellularis* bacteria in association with their outer cell membrane), was formulated in Diluvac forte adjuvant (see Example 1) at a concentration of approximately 2.8×10^8 cells per ml vaccine.

The subunit vaccine contained recombinant P1/2 and P4 as known from EP 1219711 (the 19/21 and 37 kDa proteins respectively), and the recombinant proteins expressed by genes 5074, 4320 and 5464 as described in WO2005/070958. The proteins were formulated in Diluvac forte adjuvant. The vaccine contained approximately 50 μgrams of each proteins per milliliter.

Forty 6-week-old SPF pigs were used. The pigs were allotted to 4 groups of ten pigs each. Group 1 was vaccinated once orally (at T=0) with 2 ml live "ENTERISOL® ILEITIS" (Boehringer Ingelheim) according to the instructions of the manufacturer. Group 2 and 3 were vaccinated twice intramuscularly (at T=0 and T=4w) with 2 ml of the inactivated *Lawsonia* whole cell vaccine and the recombinant subunit combination vaccine as described here-above, respectively. Group 4 was left as unvaccinated control. At T=6w all pigs were challenged orally with homogenized mucosa infected with *Lawsonia intracellularis*. Subsequently all pigs were daily observed for clinical signs of Porcine Proliferative Enteropathy (PPE). At regular times before and after challenge serum blood (for serology) and faeces (for PCR) were sampled from the pigs. At T=9w all pigs were euthanized and necropsied. Histological samples of the ileum were taken and examined microscopically.

The challenge inoculum was prepared from infected mucosa: 500 grams of infected mucosa (scraped from infected intestines) were thawed and mixed with 500 ml physiological salt solution. This mixture was homogenized in an omnimixer for one minute at full speed on ice. All pigs were challenged orally with 20 ml challenge inoculum at T=6w.

At T=0, 4, 6, 7, 8 and 9w a faeces sample (gram quantities) and a serum blood sample of each pig was taken and stored frozen until testing. The faeces samples were tested in a quantitative PCR (Q-PCR) test and expressed as the logarithm of the amount found in picograms (pg). Serum samples were tested in the commonly applied IFT test (immuno fluorescent antibody test to detect antibodies against whole *Lawsonia intracellularis* cells in the serum). For histological scoring a relevant sample of the ileum was taken, fixed in 4% buffered formalin, routinely embedded and cut into slides. These slides were stained with Hematoxylin-Eosin (HE stain) and with an immunohistochemical stain using anti-*Lawsonia intracellularis* monoclonal antibodies (IHC stain). The slides were examined microscopically. The histology scores are as follows:

HE Stain:

no abnormalities detected	score = 0
doubtful lesion	score = ½
mild lesions	score = 1
moderate lesions	score = 2
severe lesions	score = 3

IHC Stain:

no *L. intracelluaris* bacteria evident	score = 0
doubtful presence of bacteria	score = ½
presence of single/small numbers of bacteria in the slide	score = 1
presence of moderate numbers of bacteria in the slide	score = 2
presence of large numbers of bacteria in the slide	score = 3

All data were recorded for each pig individually. The score per group was calculated as the mean of the positive animals for the different parameters after challenge. The non-parametric Mann-Whitney U test were used to evaluate the statistical significance (tested two-sided and level of significance set at 0.05).

Results of Example 2

Serology

Before first vaccination all pigs were seronegative when tested for IFT antibody titres. After vaccination with the whole cell bacterin (group 2) pigs developed high IFT antibody titres whereas the controls and the pigs vaccinated with the subunit vaccine remained negative until challenge (Table 1). Two of the ENTERISOL vaccinated pigs (group 1) developed moderate IFT titres whereas all other pigs in this group remained seronegative. After challenge all pigs developed high IFT antibody titers. Mean results are depicted in table 1 (with the used dilution, 1.0 was the detection level on the lower side).

TABLE 1

Mean IFT antibody titres (2log) of pig serum after vaccination and challenge

Group	T = 0 weeks	T = 4 weeks	T = 6 weeks	T = 9 weeks
1	<1.0	1.1	1.7	>11.4
2	<1.0	3.7	>11.8	>12.0
3	<1.0	<1.0	<1.0	>11.6
4	<1.0	<1.0	<1.0	>12.0

Real-time PCR on Faeces Samples

Before challenge all faeces samples were negative. After challenge positive reactions were found in all groups. Group 1 (p=0.02), group 2 (p=0.01) and group 3 (p=0.03) had a significantly lower shedding level compared to the control. A post-challenge overview is given in table 2.

TABLE 2

Mean results of PCR on faeces samples (log pg) after vaccination and challenge

Group	T = 6 weeks	T = 7 weeks	T = 8 weeks	T = 9 weeks	Total post-challenge
1	0	1.3	3.6	1.8	6.3
2	0	0.8	2.8	1.9	5.5
3	0	0.5	3.8	2.0	5.9
4	0	0.8	4.9	4.9	10.0

Histology Scores

Group 2 had the lowest histology HE score (p=0.05), IHC score (p=0.08) and total histology score (p=0.08). The other groups had higher scores and were not significantly different from the control group. See table 3.

TABLE 3

Mean histology score for the ileum.

Group	HE score	IHC score	Total score
1	1.8	1.5	3.3
2	1.3	1.5	2.7
3	1.8	1.6	3.4
4	2.4	2.3	4.7

Conclusions with Regard to Example 2

From the results it can be concluded that the non-live whole cell *Lawsonia intracellularis* vaccine which inherently contains the carbohydrate as found also in association with the outer membrane of live *Lawsonia intracellularis* cells, induced at least partial protection. All parameters studied and histology scores were significantly or nearly significantly better compared to the controls.

Example 3

This experiment was conducted to test a vaccine comprising a substantially protein free carbohydrate containing composition as antigen. A second vaccine to be tested contained in addition to killed whole cells of *Lawsonia intracellularis*, antigens of *Mycoplasma hyopneumoniae* and Porcine circo virus (the "combi" vaccine). As a control the commercially available ENTERISOL® ILEITIS vaccine was used. Next to this, unvaccinated animals were used as a second control.

Experimental Design of Example 3

The vaccine based on a substantially protein free carbohydrate containing composition was obtained as described under Example 1.

The experimental combi vaccine contained inactivated *Lawsonia intracellularis* whole cell antigen (see Example 2 for the used method of providing the inactivated bacteria) at a level of 1.7×10^8 cells/ml. Next to this it contained inactivated PCV-2 antigen (20 μgrams of the ORF 2 encoded protein of PCV 2 per ml; the protein being expressed in a baculo virus expression system as commonly known in the art, e.g. as described in WO 2007/028823) and inactivated *Mycoplasma hyopneumoniae* antigen (the same antigen in the same dose as is known from the commercially available vaccine PORCILIS MHYO®, obtainable from Intervet, Boxmeer, The Netherlands). The antigens were formulated in a twin emulsion adjuvant "X". This adjuvant is a mixture of 5 volume parts of adjuvant "A" and 1 volume part of adjuvant "B". Adjuvant "A" consists of mineral oil droplets with an approximate average (volume weighed) size around 1 μm, stabilised with TWEEN 80 in water. Adjuvant "A" comprises 25 weight % of the mineral oil and 1 weight % of the Tween. Rest is water. Adjuvant "B" consists of droplets of biodegradable vitame E acetate with an approximate average (volume weighed) size of 400 nm, stabilised also with TWEEN 80. The adjuvant "B" comprises 15 weight % of vitamine E acetate and 6 weight % of TWEEN 80, rest is water.

Sixty-four 3-day-old SPF piglets were used. The pigs were allotted to four groups of 14 piglets and one group of 8 piglets (Group 4). Group 1 was vaccinated intramuscularly at 3 days of age with 2 ml of the combi vaccine, followed by a second vaccination at 25 days of age. Group 2 was vaccinated intramuscularly once with 2 ml combi vaccine at 25 days of age. Group 3 was vaccinated orally with 2 ml ENTERISOL®ILEITIS (Boehringer Ingelheim) at 25 days of age according to prescriptions. Group 4 was vaccinated intramusculary at 3 and 25 days of age with 2 ml of the non-protein carbohydrate vaccine. Group 5 was left unvaccinated as a challenge control group. At 46 days of age all pigs were challenged orally with homogenized infected mucosa. Subsequently all pigs were daily observed for clinical signs of Porcine Proliferative Enteropathy (PPE). At regular times before and after challenge serum blood and faeces samples were taken from the pigs for serology and PCR respectively. At 68 days of age all pigs were euthanized and post-mortem examined. The ileum was examined histologically.

The other issues in the experimental design were the same as described in Example 2, unless indicated otherwise.

Results of Example 3

Serology

1—Lawsonia

Before first vaccination all pigs were seronegative for IFT antibody titres. After vaccination with the combi vaccine (groups 1 and 2) and the non-protein carbohydrate vaccine (group 4), many pigs developed IFT antibody titres whereas the controls and the pigs vaccinated with ENTERISOL remained seronegative until challenge. After challenge all pigs (except two in the ENTERISOL group) developed IFT antibody titres. For an overview of the mean values obtained,

see table 4 (due to the higher dilution when compared to example 2, the detection level was 4.0).

TABLE 4

Mean IFT *Lawsonia* antibody titres (2log) of pig serum after vaccination and challenge

Group	T = 3 days	T = 25 days	T = 46 days	T = 67 days
1	<4.0	<4.0	7.9	10.3
2	<4.0	<4.0	4.8	9.8
3	<4.0	<4.0	<4.0	8.5
4	<4.0	<4.0	6.9	10.6
5	<4.0	<4.0	<4.0	9.0

2—*Mycoplasma hyopneumoniae*

With respect to Mhyo, at the start of the experiment as well as day of booster (25-day-old) all pigs were seronegative for Mhyo. After booster vaccination group 1 developed high Mhyo antibody titres, at the same level of those obtained with the commercially available prime-boost vaccine PORCILIS MHYO®. The results are given in Table 5 below. Apparently, under these circumstances (given intramuscularly) only when a booster vaccination is given the antibody titres at 46 days are above detection level (6.0 for the method used). It is known however that a single shot vaccination with Mhyo antigens may provide sufficient protection, in particular when administered intradermally (see e.g. WO 2007/103042).

TABLE 5

Mean IFT Mhyo antibody titres (2log) of pig serum after vaccination

Group	T = 3 days	T = 25 days	T = 46 days
1	Below detection level	Below detection level	8.3
2	Below detection level	Below detection level	Below detection level
5	Below detection level	Below detection level	Below detection level

3—Porcine Circo Virus

With respect to PCV, at 3-day-old the piglets had high maternally derived PCV antibody titres. At day of booster (25-day-old) the vaccinates (group 1) had a similar titre compared to group 2 and the control group. The PCV titre at 25-day-old was slightly lower compared to the titre at 3-day-old. After the vaccination at 25-day-old the titres of group 1 (2 vaccinations: at day 3 and 25) and group 2 (one vaccination at day 25) remained at a high level whereas control piglets showed a normal decrease in maternally derived antibodies. The PCV titres obtained are comparable to the titres obtainable with a single vaccine containing the same antigen (e.g. Intervet's Circumvent PCV, which vaccine provides very good protection against PCV). For an overview of the mean values, see the table given below.

TABLE 6

Mean IFT PCV antibody titres (2log) of pig serum after vaccination

Group	T = 3 days	T = 25 days	T = 46 days
1	11.5	9.6	10.1
2	12.1	9.5	10.8
5	10.9	9.1	7.0

Real-time PCR on Faeces Samples

Three weeks after challenge, pigs of group 1, 2 and 4 had less *Lawsonia* (DNA) in their feces compared to groups 3 and 5. Only the differences between group 1 and 3 (ENTERISOL) and group 4 and 3 were statistically significant ($p<0.05$, Mann-Whitney U test). For the mean results, see table 7.

TABLE 7

Mean results of PCR on faeces samples (log pg) after vaccination and challenge

Group	Mean value
1	1.0
2	1.2
3	2.0
4	0.6
5	1.8

Histological Scores

Histology scores of group 1 and 4 were significantly lower compared to those of groups 3 and 5 ($p<0.05$, two-sided Mann-Whitney U test (see table 8). The number of pigs with confirmed PPE were 2/13 in group 1, 6/12 in group 2, 12/14 in group 3, 2/7 in group 4 and 12/14 in the control group 5. Groups 1 and 4 had a significantly lower incidence of PPE compared to groups 3 and 5 ($p<0.05$, two-sided Fischers' exact test).

TABLE 8

Mean histology score for the ileum.

Group	HE Score	IHC Score	Total Score
1	0.4	0.6	1.0
2	0.7	0.7	1.4
3	1.6	1.4	3.0
4	0.4	0.4	0.8
5	1.9	1.5	3.4

Conclusion of Example 3

From the results it can be concluded that the carbohydrate outer cell membrane antigen offers a relatively good protection against ileitis. It is also found that the whole cell *Lawsonia* bacterin is a good means of offering the carbohydrate antigen in a vaccine to combat ileitis. Moreover, given the fact that the combination vaccine provided titres for

Mhyo and PCV antibodies to a level comparable with the levels obtainable with available single vaccines that are adequate to combat these micro-organisms, it has been demonstrated that a combination vaccine comprising non-live *Lawsonia intracellularis* antigens in combination with Mhyo and PCV antigens is suitable to combat *Lawsonia intracellularis* as well as *Mycoplasma hyopneumoniae* and Porcine circo virus.

The invention claimed is:

1. A method for protecting a swine against virulent challenge by *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine Circo Virus infection comprising systemically administering to the swine a non-live combination vaccine comprising an immunogenically effective amount of non-live antigens of *Lawsonia intracellularis, Mycoplasma hyopneumoniae* and Porcine Circo Virus, and a carrier, wherein the non-live *Lawsonia intracellularis* antigens comprise a carbohydrate found in live *Lawsonia intracellularis* cells in association with the outer cell membrane of those cells, and, wherein the vaccine comprises an oil in water adjuvant containing oil droplets of sub-micrometer size.

2. The method according to claim 1, wherein the adjuvant comprises droplets of biodegradable oil and droplets of mineral oil, the droplets of biodegradable oil having an average size that differs from the average size of the droplets of mineral oil.

3. The method according to claim 2, wherein the *Lawsonia intracellularis* antigens comprise material resulting from the killing of *Lawsonia intracellularis* bacteria.

4. The method according to claim 3, wherein the *Lawsonia intracellularis* antigens comprise killed whole cells of *Lawsonia intracellularis* bacteria.

5. The method according to claim 1, wherein the *Lawsonia intracellularis* antigens comprise material resulting from the killing of *Lawsonia intracellularis* bacteria.

6. The method according to claim 5, wherein the *Lawsonia intracellularis* antigens comprise killed whole cells of *Lawsonia intracellularis* bacteria.

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